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Originals

Artificial Syntheses of Mycobionts of *Usnea confusa* ssp. *kitamiensis* and *Usnea orientalis* with Their Natural and Non-Natural Phycobiont

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ツブコナサルオガセ（*Usnea confusa* ssp. *kitamiensis*）・ハナサルオガセ（*Usnea orientalis*）の共生菌と同種および異種地衣類の共生藻からの地衣体の再合成

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Resynthesis studies revealed that the mycobionts *Usnea confusa* ssp. *kitamiensis* and *Usnea orientalis* fully lichenized with their natural phycobionts. In addition, *U. confusa* mycobiont synthesized lichens with non-natural phycobionts derived from different genera such as *Sulcaria* and *Parmotrema*. The fibrils formed on synthetic cultures of *U. confusa* ssp. *kitamiensis* and *U. orientalis* mycobionts with non-natural phycobionts were similar to those formed on synthetic cultures of the natural bionts, except for differences in the developmental degree of axis, coverage of mucilaginous substances on the surface of fibrils, thickness and length of fibrils and frequency of fibril formation.

Although HPLC showed that no secondary products were present in the synthetic culture of *U. confusa* ssp. *kitamiensis* mycobiont with *U. orientalis* phycobiont, crystals, which were presumed secondary products, were seen on the surface of the fibrils formed. Usnic acid was demonstrated in colonies formed by *U. orientalis* mycobiont alone, whereas it was not positively demonstrated in clusters formed by *U. confusa* ssp. *kitamiensis* mycobiont without phycobiont. Usnic and salazinic acids were demonstrated in synthetic cultures of *U. orientalis* mycobiont with its natural phycobiont as well as with *U. confusa* ssp. *kitamiensis* phycobiont.

Introduction

Recent synthetic studies of lichens have enabled us to observe how they develop under laboratory conditions. Lichen belonging to species of *Cladonia* and *Usnea* can be synthesized artificially in flasks or tubes (Ahmadjian et al. 1980, 1981, 1985; Yamamoto et al. 1991). As previously reported by Kon et al. (1990), thalli of *Usnea confusa* ssp. *kitamiensis* can be routinely induced from their growing tissues *in vitro* and the induced thalli are quite similar to natural ones with respect to morphology and chemistry. Ahmadjian et al. (1980, 1982, 1987), however, reported that a mycobiont isolated from a lichen assumed a compact elevated form which was unlike that of the natural thallus. These authors also reported that mycobionts of *Cladonia cristatella* and *Usnea strigosa* could lichenize species of *Trebouxia* other than their natural phycobionts. Exactly, how a phycobiont influences specific morphogenetic and chemical responses of the mycobiont is not yet known.

Materials and methods

Usnea confusa ssp. *kitamiensis* Asah. (abbreviated as *U. confusa* in this paper) was collected on Mt. Tanzawa, Kanagawa Prefecture in May of 1987 and *U. orientalis* Asah. on Mt. Kuji, Ooita Prefecture in May of 1989. Mycobionts and phycobionts were obtained by the following procedures:

1. Growing tissues were obtained from thalli of these lichens by the method proposed by Yamamoto et al. (1985).

2. Actively growing tissues were aseptically put into a sterilized mortar and then homogenized with sterilized distilled water.

3. The suspensions thus obtained were spread over plate media by means of a spreader.

4. Mycobiont and phycobiont colonies that developed separately on the plates after one month were inoculated onto slant media in different test tubes. The medium was composed of 2% w/v malt extract, 0.2% w/v yeast extract and 2% w/v agar.

5. Bionts thus isolated were used for two series of synthetic experiments described below.

Phycobionts isolated from *Sulcaria sulcata* Bystr. ex Brodo et Haksw., *Parmotrema tinctorum* (Nyl.) Hale, *Ramalina litoralis* Asha., by the procedure mentioned above were also used for the synthetic studies. Those phycobionts were identified as follows by Dr. T. Nakano. *Sulcaria sulcata* phycobiont: *Trebouxia jamsii* (Hildreth et Ahmadjian) Gärtner

Parmotrema tinctorum phycobiont: *Trebouxia sp.*

Ramalina litoralis phycobiont: *Trebouxia gigantea* (Hildreth et Ahmadjian) Gärtner

Usnea confusa phycobiont: *Trebouxia potteri* Ahmadjian ex Gärtner

Usnea orientalis phycobiont: *Trebouxia irregularis* Hildreth et Ahmadjian

The first series of synthetic experiments was conducted between the mycobionts of *U. confusa* and *U. orientalis* and their natural phycobionts. Sterile techniques were used in all of the studies. Cultured hyphae of the mycobiont were homogenized in water with a morter and pestle and then mixed with its natural phycobiont. The mixture of bionts was then cultured under optimum conditions (Kon et al. 1990). After 3.5 months, the mixed culture was observed with both light and scanning electron microscopes. The first series of synthetic experiments served as the control in the present study. In the second series of synthetic experiments, the mycobionts of *U. confusa* and *U. orientalis*, were tested with various phycobionts derived from different species or genera of lichens.

As previously reported by Kon et al. (1990), thalli induced from growing tissue of *U. confusa*

Table 1. Comparison of synthetic cultures of *U. confusa* mycobiont.

	with *U. confusa* phycobiont (control)	with *U. orientalis* phycobiont	without phycobiont
fibril formation rate (%)	100	60	
number of fibrils (mean ± SD)	114.2 ± 16.1	12.6 ± 7.1	
length of fibrils (μm) (mean ± SD)	1175 ± 56.5	808 ± 130.5	
usnic acid*	72.7	not detected	not detected
norstictic acid*	85.4	not detected	not detected
salazinic acid*	not detected	not detected	not detected

*secondary products content (μg/g.dr.wt.)

always formed fibrils similar to those of the natural thalli. To examine the influence of various phycobionts on one mycobiont and/or to determine the range of suitable phycobionts for one mycobiont, synthetic cultures were observed as follows: Fibril formation rate was calculated as a percentage of test tubes, in which fibrils or at least one fibril were formed, to ten test tubes tested. Number of fibrils was indicated as an average of fibrils per one test tube. Length of fibril (μm) was calculated to be an average length of five best developed fibrils in a test tube. Secondary products of each synthetic culture were determined by TLC (Culberson & Johnson 1982) and HPLC. HPLC was performed using a JASCO HPLC system equipped with a Finepack SIL ODS column (4.6 mm × 150 mm). After crude acetone extracts were filtered through Chromatodisk (0.45μm), secondary products were applied to HPLC, and eluted with $MeOH/H_2O/H_3PO_4$ (90:10:2). The compounds were monitored by the detection at 230 or 240 nm and flow-rate was maintained at 1.0 ml/min. The retention time of each compounds in samples was checked by using authentic compounds under specified conditions. The amounts of compounds were determined by their peak areas in comparison with appropriate standards.

Results

Three and half months after inoculation, syn-

Table 2. Comparison of synthetic cultures of *U. orientalis* mycobiont.

	with *U. orientalis* phycobiont (control)	with *U. confusa* phycobiont	without phycobiont
fibril formation rate (%)	100	90	
number of fibrils (mean ± SD)	56.0 ± 35.4	14.2 ± 0.5	
length of fibrils (μm) (mean ± SD)	1154 ± 49.6	675 ± 118.6	
usnic acid*	78.5	84.5	100.8
salazinic acid*	1125.3	116.9	not detected

*secondary products content (μg/g.dr.wt.)

thetic cultures of mycobionts of *U. confusa* and *U. orientalis* without phycobionts yielded irregular pale brown mycelia. Although these mycobionts grew about ten times their initial weight, no fibrils was formed on their mycelial clusters. The results of HPLC tests showed that they produced neither salazinic nor norstictic acids, as substances produced in the natural thalli of these lichens. A small amount of usnic acid, a cortical substance in both species of lichens, was detected in colonies of *U. orientalis* mycobiont but not in those of *U. confusa* mycobiont (Tables 1, 2).

In the first series of synthesis experiments, mycobionts of *U. confusa* and *U. orientalis* fully lichenized with their natural phycobionts and fibrils were found in all test tubes examined. In other words, the fibril formation rate was 100%. These fibrils were quite similar to those of thalli induced from growing tissues as well as those of natural thalli of lichens (Kon et al. 1990). Usnic acid and norstictic acids were demonstrated in clusters of *U. confusa* mycobiont with its natural phycobiont, and usnic and salazinic acids were identified in clusters of *U. orientalis* mycobiont with its natural phycobiont.

In the second series of synthesis experiments, each mycobiont of two *Usnea* species was cultured with phycobionts isolated from different species of lichens. The results on the second series of synthesis experiments were compared with those of the first series. The results were as follows.

1. Synthetic culture of *U. confusa* mycobiont with

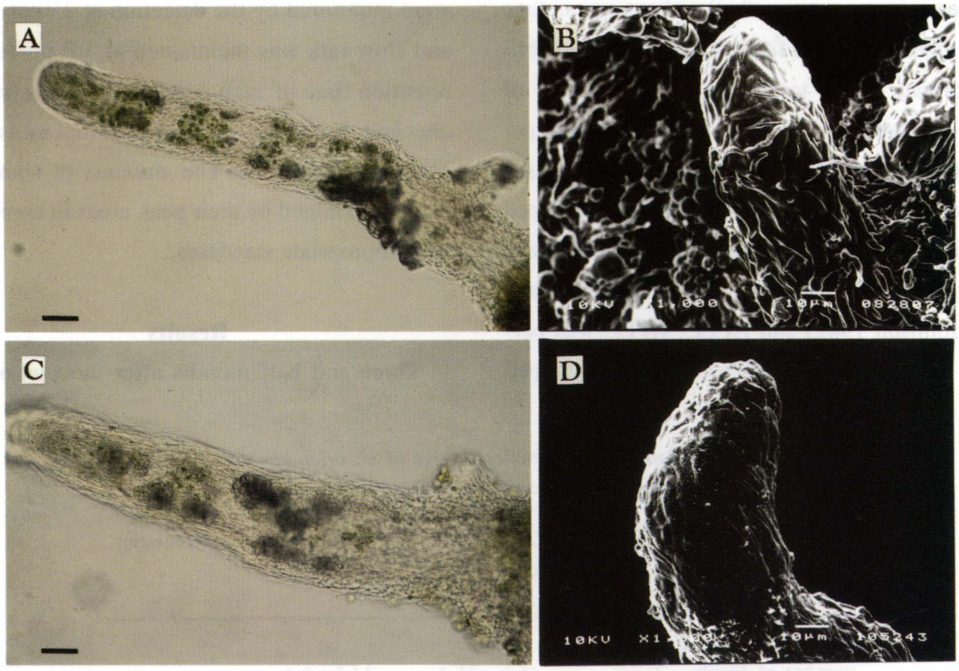

Fig. 1. Fibrils formed by *U. confusa* mycobiont
A, B: fibril formed on synthetic culture of *U. confusa* mycobiont with its natural phycobiont.
C, D: fibril formed on synthetic culture of *U. confusa* mycobiont with *U. orientalis* phycobiont.
The cortex, gonidial layer, medulla, and axis are well differentiated in A and C (light micrograph), and the surface is covered with gelatinous substances in B and D (scanning electron micrograph).
Scale bar=50μm (A, C), 10μm (B, D)

U. orientalis phycobiont (*Trebouxia irregularis*)

U. confusa mycobiont fully lichenized with the *U. orientalis* phycobiont. After 3.5 months, the formation of many upright fibrils were recognized in synthetic culture. The fibrils formed were cylindrical and more or less irregular in thickness, but were shorter (808 μm) than those of the control culture (1175 μm) (Table 1). In cross section of fibrils, algal cells (phycobiont cell) were scattered through the medulla and were 3 μm in diameter near the tip of fibrils, and 3–11 μm in diameter near the base. The axis composed of longitudinally running hyphae was formed through the base to the tip of each fibril. The cortex was rather thin (about 5 μm thick) and composed of thin-walled hyphae coated with mucilaginous substances as reported by Ahmadjian and Jacobs (1982) and Kon et al. (1990) (Fig. 1A–D).

Even though many fibrils were formed on synthetic cultures of *U. confusa* mycobiont with *U. orientalis* phycobiont, the fibril formation rate was 60%, whereas that of the control culture was 100%. An average number of fibrils formed in the present synthetic culture was 12.6 versus 114.2 in the control (Table 1).

Although usnic and norstictic acids were produced in synthetic cultures of *U. confusa* with its natural phycobiont, no secondary products were detected in the present synthetic culture. However, two types of crystals were found on the surface of fibrils; they were pale yellow needles (2.5 μm thick and 15 μm long) and colorless thin plate clusters (Fig. 1A, C). In shape and color, they were similar to crystals of usnic and norstictic acids, respectively, as reported on the surface of synthetic fibrils of *Usnea strigosa* (Ahmadjian and Jacobs 1985). Unfortunately, these crystals have not yet been positively identified probably because of their trace amounts.

2. Synthetic culture of *U. orientalis* mycobiont with *U. confusa* phycobiont (*Trebouxia potteri*).

While the average number of fibrils formed on a synthetic culture of *U. orientalis* mycobiont with its natural phycobiont (control) was 56.0, that of *U. orientalis* mycobiont with the *U. confusa* phycobiont was smaller (14.2) (Table 2). However, fibril formation rates of the two types of culture were very similar i.e., 90% versus 100% (Table 2). Fibrils formed on synthetic cultures of *U. orientalis* mycobiont with *U. confusa* phycobiont were shorter (675 μm in average) and irregular in thickness, control fibrils were longer (1154 μm in average) and cylindrical. In cross section of fibrils, algal cells (phycobiont cells) were scattered in the medulla. They were about 2.5 μm in diameter near the tip of a fibril and 3–11 μm in diameter near the base. The axis of the fibrils was poorly differentiated (about 20 μm thick at the middle; Fig. 2C) while the cortex was thin (about 5 μm thick) and composed of thin-walled hyphae coated with mucilaginous substances except at the base (Fig. 2D).

In the present synthetic culture the same as control, both usnic and salazinic acids were produced in natural thalli of *U. orientalis*, even though only usnic acids was produced in culture of *U. orientalis* mycobiont alone (Table 2). When taking into consideration the amount of usnic acid produced by the *U. orientalis* mycobiont alone, it is noteworthy that the amount of usnic acid had decreased in the synthetic culture of *U. orientalis* mycobiont with its natural phycobiont as well as the culture with non-natural phycobionts, in which salazinic acid was produced (Table 2).

3. Synthetic cultures of *U. confusa* mycobiont with phycobionts isolated from different species of lichens.

Synthetic cultures were made of *U. confusa* mycobiont with phycobionts isolated from *Sulcaria*

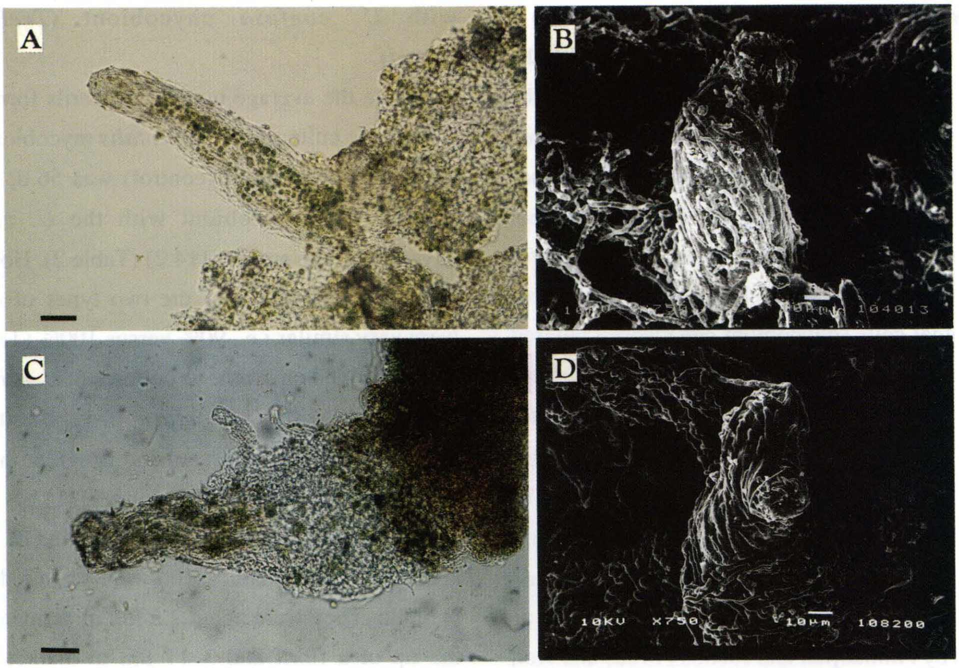

Fig. 2. Fibrils formed by *U. orientalis* mycobiont
A, B: fibril formed on synthetic culture of *U. orientalis* mycobiont with its natural phycobiont.
C, D: fibril formed on synthetic culture of *U. orientalis* mycobiont with *U. confusa* phycobiont.
Axis is well developed in A but is poor in C (light micrograph). The surface is covered with gelatinous substances in B but is nacked near the base in D (scanning electron micrograph).
Scale bar=50μm (A, C), 10μm (B, D)

sulcata, Parmotrema tinctorum and *Ramalina litoralis*. fibril were not formed on synthetic cultures of *U. confusa* with *R. litoralis* phycobiont, but they were formed on synthetic cultures of *U. confusa* mycobiont with the other phycobionts (Fig. 3A, C). Fibrils formed in these synthetic cultures were similar to those from synthetic cultures of *U. confusa* mycobiont and its natural phycobionts (control). They were, however, more or less irregular in cross section, 120–130 μm in diameter at the middle part and poorly covered with mucilaginous substances. While the fibril formation rates in these synthetic cultures were lower than those of the control, the rate in culture with *S. sulcata* phycobiont was almost the same as that of culture of *U. confusa* mycobiont with its natural phycobiont. Each average number and average length of fibrils formed on synthetic cultures of *U. confusa* mycobiont with *S. sulcata* and *P. tinctorum* phycobionts were lower than the those of control. Norstictic acid was detected in synthetic culture of *U. confusa* mycobiont and *P. tinctorum* phycobiont. Otherwise, no secondary products were detected (Table 3).

Discussion

The question of how specific particular fungal symbiont (mycobiont) is to its algal partner (phycobiont) is subject of active discussion among present-day lichenologists. In the present study, we attempted to synthesize two lichen fungi, mycobionts of *U. confusa* and *U. orientalis*, with their natural phycobionts and phycobionts isolated from different species of lichens. The results obtained

Table 3. Comparison of synthetic cultures of *U. confusa* mycobiont with various phycobionts.

	with *Sulcaria sulcata* phycobiont	with *Parmotrema tinctorum* phycobiont
fibril formation rate (%)	90	40
number of fibrils (mean ± SD)	9.3 ± 4.1	12.5 ± 1.7
length of fibrils (μm) (mean ± SD)	350 ± 75	1060 ± 227
usnic acid*	not detected	not detected
norstictic acid*	not detected	16.7
salazinic acid*	not detected	not detected

*secondary products content (μg/g.dr.wt.)

have revealed that the *Usnea* mycobionts could fully lichenize their natural phycobiont and always produced many fibrils similar to natural thalli. They also were able to lichenize non-natural *Trebouxia* phycobionts isolated from different species of lichens, except for the *Trebouxia* phycobiont isolated from *Ramalina litoralis.* Fibril formation rates varied by the combinations of mycobiont and phycobiont, being higher in synthetic cultures of mycobionts with phycobionts isolated from the same genus of lichens. However, it was also high in synthetic cultures of *U. confusa* mycobiont with *S. sulcata* phycobiont, probably because *Sulcaria* and *Usnea* belong to the same family, *Usneaceae.* In contrast, it was lower (40%) in synthetic cultures of *U. confusa* mycobiont and *P. tinctorum* phycobiont, probably because these two lichens belong to different family and are not related to each other. It should be noted that the axis was poorly developed and the surface not covered with mucilaginous substances in fibrils formed on synthetic cultures of *U. confusa* mycobiont with *S. sulcata* and *P. tinctorum* phycobionts. Therefore, these bionts were not preferable in lichenization with the *U. confusa* mycobiont. These facts indicate the following; *Usnea* mycobionts have some specificity in lichenizing a phycobiont and may easily lichenize phycobionts isolated from closely related lichens. In external and internal morphology and in size, fibrils formed in synthetic cultures of *U. confusa* mycobiont with various phycobionts were quite similar to those formed from synthetic culture of the mycobiont with its natural phycobiont. However, in synthetic culture of *U. confusa* mycobionts with phycobionts of *S. sulcata* and *P. tinctorum*, fibrils were not round in cross section and the average number of fibrils was fewer.

It has been generally accepted that the phycobiont provides photosynthetic products for the mycobiont. However, how a phycobiont influences a specific morphogenetic response of the mycobiont is not known. The present study revealed that mycobionts of *U. confusa* and *U. orientalis* yielded only irregular mycelial colonies in the absence of phycobionts. In contrast, synthetic culture of these mycobionts with phycobionts, not necessarily with thei natural phycobiont, formed fibrils similar to those of natural lichen thalli, with one exception. This fact indicates that the phycobiont plays a significant role in the morphogenesis of lichenized fungi.

Culberson et al. (1980) is of opinion that one of the most enigmatic problem in experimental

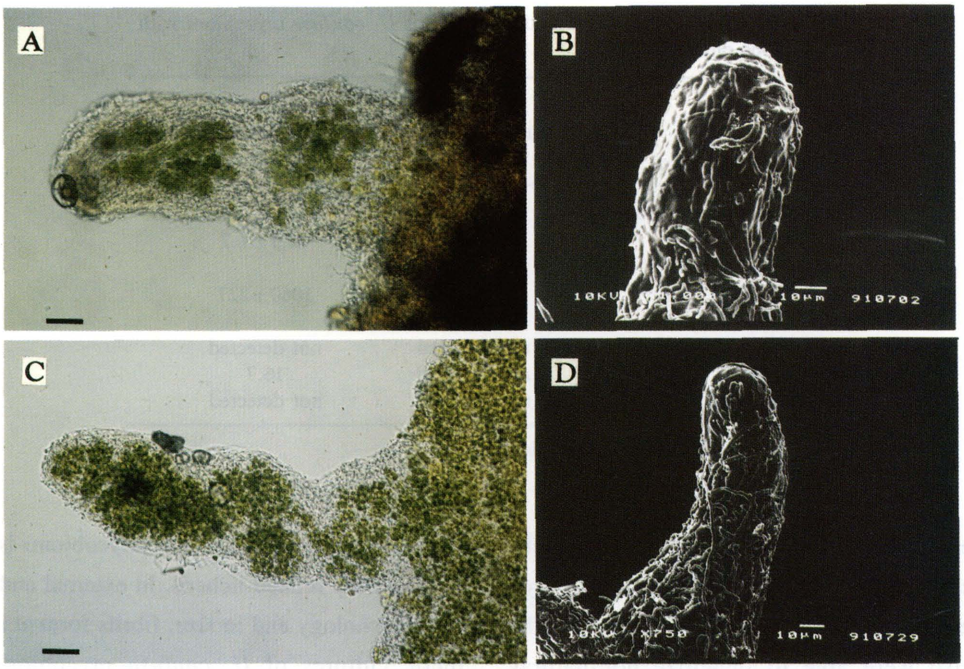

Fig. 3. Fibrils formed by *U. confusa* mycobiont with phycobiont isolated from different species of lichens.
A, B: fibril formed on synthetic culture of *U. confusa* mycobiont with *S. sulcata* phycobiont.
C, D: fibril formed on synthetic culture of *U. confusa* mycobiont with *P. tinctorum* phycobiont.
Axis is poorly developed in A and C (light micrograph), and the surface is poorly covered with gelatinous substances in B and D (scanning electron micrograph).
Scale bar=50μm (A, C), 10μm (B, D)

lichenology has been the failure of the separate mycobionts to synthesize in culture the same chemical compounds they do when they are in symbiosis with algae. Furthermore, he concluded that lichen substances were not produced by the culture of mycobionts alone, but synthesized by the symbiotic culture with algae. Recently Culberson et al. (1992) succeeded in induction of a lichen specific secondary pathway in the fungi isolated from their photosynthetic partners. In the present study, usnic acid was demonstrated in cultures formed by *U. orientalis* mycobiont alone which was cultured in the malt-yeast extract medium. Usnic and norstictic acids were demonstrated in synthetic cultures of *U. confusa* mycobiont with its natural phycobiont. In addition, norstictic acid was also produced even in synthetic culture of *U. confusa* mycobiont with *P. tinctorum* phycobiont. Usnic and salazinic acids were demonstrated in synthetic cultures of *U. orientalis* mycobiont with *U. confusa* phycobiont as well as its natural phycobiont. It should be noted that *U. confusa* mycobiont produced norstictic acid and usnic acid when it was cultured with preferable phycobionts, but not salazinic acid. However, *U. orientalis* mycobiont produced salazinic acid along with usnic acid when it was cultured with its natural phycobiont or with *U. confusa* phycobiont, but never produced norstictic acid. This fact seems to indicate that every mycobiont isolated from a lichen preserves its proper productivity of the same substances found in the original lichen. The productivity may be or not may be in dormancy when the mycobiont was cultured without phycobiont or with unpreferable phycobiont. When the mycobiont is cultured alone, biosynthetic pathway of proper lichen substances may be blocked and then the dissimilation products may follow into different pathways to produce different substances which are not found in the lichen. However, the mycobiont may display its productivity whenever it meet with a condition to lichenize with a prefered phycobiont.

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要 旨

ツブコナサルオガセ（*Usnea confusa* ssp. *kitamiensis*）とハナサルオガセ（*Usnea orientalis*）の両種から得た共生体（菌・藻）を相互に交換しても地衣体の再合成が観察できた．加えて，*Usnea confusa* の共生菌は *Sulcaria* 属や *Parmotrema* 属から得た共生藻でも地衣体の再合成がみられた．しかし，異種の地衣類から得られた共生藻の場合では，再合成した地衣体は自身の共生藻の場合の地衣体と比較して，軸分化・地衣体表面の粘液性物質・地衣体の長さおよび地衣体形成の頻度に違いがみられた．

Usnea orientalis 共生菌のみからなる培養組織にはウスニン酸が検出されたが，*Usnea confusa* 共生菌には二次代謝産物は検出されなかった．また，*Usnea confusa* 共生菌と *Usnea orientalis* 共生藻からなる培養組織からは二次代謝産物は検出されなかったが，分化した地衣体表面には二次代謝産物と考えられる結晶がみられた．しかし，*Usnea confusa* 共生菌と *Parmotrema tinctorum* 共生藻からなる培養組織からはノルスチクチン酸が検出された．また，*Usnea orientalis* 共生菌と *Usnea confusa* 共生藻からの培養組織には，ウスニン酸のほかサラチン酸も検出できた．